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TITLE: A Novel Approach for Effectively Treating SCI Pain, Improving Opioid Efficacy, and Preventing Opioid-Induced Constipation: Key Role of Toll-Like Receptor 4 (TLR4)

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14. ABSTRACT
Spinal cord injury (SCI) is a disabling and costly condition affecting wounded military personnel (1). SCI is also one of the leading causes of central neuropathic pain, and military personnel that sustain SCI in the field by improvised explosive devices (IEDs), grenades, gunshot wounds, etc. are at an even greater risk of developing chronic pain as well as emotional symptoms due to the polytraumatic nature of these injuries (2). Furthermore, central neuropathic pain in general is often intractable to treatment; current therapies including opioids only provide ~50% pain relief in 1 out of 2-3 people (3) and the therapies are even less effective in military blast SCI due to the complexity of the injury (4). This level of treatment efficacy is unacceptable for war fighters, military personnel, veterans, and citizens as a whole. SCI patients are almost universally treated with opioids as a first-line treatment, but recent evidence in the animal literature and recently in the clinical literature indicates that opioid administration after traumatic injury can have deleterious consequences. This proposal will test a clinically relevant therapeutic, (+)-naltrexone, that we predict will improve the efficacy of opioids for controlling SCI below-level pain while decreasing the negative consequences of opioid use. We predict that the mechanism by which (+)-naltrexone exerts at least some of its effects is via toll-like receptor 4. Thus our aim is to improve the quality of life for service-members, veterans, caretakers, and the general population.

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INTRODUCTION

Spinal cord injury (SCI) is a disabling and costly condition affecting wounded military personnel[1]. SCI is also one of the leading causes of central neuropathic pain, and military personnel that sustain SCI in the field by improvised explosive devices (IEDs), grenades, gunshot wounds, etc. are at an even greater risk of developing chronic pain as well as emotional symptoms due to the polytraumatic nature of these injuries[2]. Furthermore, central neuropathic pain in general is often intractable to treatment; current therapies including opioids only provide ~50% pain relief in 1 out of 2-3 people[3] and the therapies are even less effective in military blast SCI due to the complexity of the injury[4]. This level of treatment efficacy is unacceptable for war fighters, military personnel, veterans, and citizens as a whole. SCI also causes bowel dysfunction ("neurogenic bowel dysfunction"), which is characterized by constipation and/or fecal incontinence[5]. SCI patients are almost universally treated with opioids as a first-line treatment, but recent evidence in the animal literature and recently in the clinical literature indicates that opioid administration after traumatic injury can have deleterious consequences (e.g., exacerbating chornic pain and constipation). This proposal will test a clinically relevant therapeutic, (+)-naltrexone, that we predict will improve the efficacy of opioids for controlling SCI below-level pain while decreasing the negative consequences of opioid use. We predict that the mechanism by which (+)-naltrexone exerts at least some of its effects is via toll-like receptor 4. Thus our aim is to improve the quality of life for service-members, veterans, caretakers, and the general population.

Keywords
Spinal cord injury, central neuropathic pain, rat, opioids, morphine, (+)-naltrexone, analgesia, allodynia, hyperalgesia, toll-like receptor 4

OVERALL PROJECT SUMMARY

Task 1. Obtain approval from the University of Colorado Institute Animal Care & Use Committee
(IACUC) for all animal work in the proposal. (Timeframe: 1-2 months prior to project start).
2a. Submit animal protocol covering all animal work at Boulder for IACUC meeting at least 2 months prior to anticipated start date (here, assumed to be September 1, 2013), hence July 2013 meeting or before. If revision required, submit for August 2013 meeting at latest.

Task 1 has been completed.

Milestone 1: Animal protocol is approved to allow funding to be received & to allow the project to start.

Milestone 1 has been completed.

Task 2. Purchase of, and corresponding training for operation of and maintenance of the Infinite Horizon impactor, for contusion spinal cord injury.
2a. Purchase of MASCIS (submission of sole source letter, ordering of equipment, and awaiting delivery).
2b. Team member will attend the training offered by W. M. Keck Center for Collaborative Neuroscience.

Task 2a Progress. Infinite Horizon spinal cord impactor (state-of-the-art in the SCI field) was purchased and assembled.
Task 2b Progress. Hired an expert in January 2015 for completing SCI surgeries. Dr. Andrew Gaudet, a Postdoctoral Research Associate, trained at The Ohio State University where he learned everything taught in the renowned Spinal Cord Injury Training Program. Since Gaudet already has training in SCI research, further training courses were not required. Since arriving, he assembled the device and has successfully run multiple rat and mouse contusion SCI experiments.

Milestone 2: Obtain the MASCIS impactor, as well as enhanced training using the MASCIS impactor, and knowledgeable on caring for, and maintaining of, the equipment.

MILESTONE 2 IS COMPLETE. Rather than purchasing the MASCIS impactor, the gold standard impactor for SCI research (Infinite Horizon) was purchased and assembled. It is now in regular use.

Task 4. Training on performing contusion spinal cord injury.
4a. Team member to attend the Spinal Cord Injury Research Program Course at The Ohio State University.

Task 4a Progress. By hiring Gaudet, an experienced researcher from Ohio State with training on all aspects taught at the Spinal Cord Injury Training Program, the expense of sending a trainee to the course was avoided. Task 4a is complete.

MILESTONE 4 IS COMPLETE. Gaudet and his team of research assistants (Professional Research Assistant and undergraduates) have all the resources and expertise to perform contusion SCI surgery, behavior, and all other proposed experiments.

<u>Task 6.</u> Aim III: Do robust phenomena from above generalize to the classic, widely employed SCI contusion model?
6b. Early co-administration of morphine and (+)-naltrexone: Contusion surgery and testing (only robust differences in above experiments will be pursued, and may be Hargreaves, motor function and/or constipation during the first week and co-administration of morphine and (+)-naltrexone starting 1 or 24 hr post surgery; von Frey and motor testing weekly starting on day 8; Unblinding of data & data analysis) (Timeframe: January 2015 – September 2015)

Task 6b. Progress. In our previous reports, we outlined that there were issues with studying constipation, and with collecting consistent results with morphine and (+)-naltrexone treatment after SNAP surgery. After Gaudet's arrival, research has focused mainly on establishing the clinically-relevant contusion SCI model. Thus, our model aimed to identify several processes: 1. A rat SCI model of neuropathic pain; 2. A set-up to study fecal production/constipation after SCI; and 3. Whether morphine influenced pain and fecal production after SCI (as hypothesized). Once these key parameters were established in a useful model, we could then focus on improving morphine-related challenges (i.e., chronic pain and constipation) using (+)-naltrexone.

Establishing a neuropathic pain model
Prior to starting morphine/(+)-naltrexone SCI experiments, we sought to confirm the effectiveness of SCI pain models in our own lab. In initial experiments, we attempted to establish an effective contusion SCI model to confirm the expression of SCI-induced neuropathic pain that other laboratories have reported[6-8].

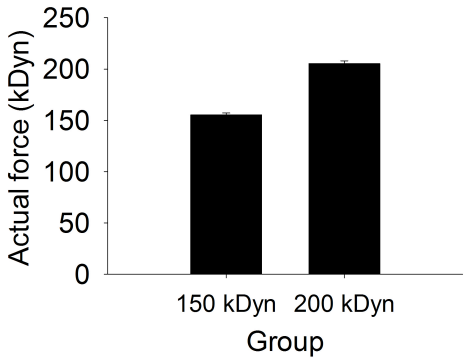

Fig. 1. Actual contusion SCI forces from moderate (150 kDyn; n=9) and severe (200 kDyn; n=8) groups.

Based on this previous research (citations above), we expected that moderate (150-kDyn force) or severe (200-kDyn force) midline contusion SCI could cause below-level neuropathic pain. Therefore, our initial experiment included three groups: sham (laminectomy only; n=8), moderate SCI (n=9), and severe SCI (n=8). Sprague-Dawley rats were subjected to T9/T10 laminectomy, then were stabilized and raised slightly on the device's stereotaxic frame. SCI rats were leveled to ensure midline contusion, then placed under the Infinite Horizon impactor for contusion SCI (Fig. 1).

Our neuropathic pain outcomes were mechanical allodynia (von Frey filament testing) and thermal hyperalgesia (Hargreaves plantar hindpaw heat) (Fig. 2). For behavioral tests, all experimenters are blinded to experimental group. Rats underwent pre-surgical testing for nociceptive thresholds (2x for each von Frey and Hargreaves tests). Thresholds were tested weekly post-injury until the final timepoint (77 dpi).

As predicted, SCI-induced below-level neuropathic pain was somewhat delayed and became apparent ~14-35 dpi. This was at least partly due to the lack of sensory and motor control at early times post-injury (particularly in the 200-kDyn group). Significant neuropathic pain was observed, particularly in the severe 200-kDyn group; however, these scores were complicated somewhat by SCI-induced sensorimotor deficits in severely injured rats. The moderate SCI group showed thermal, but not mechanical hypersensitivity, but did not have confounds related to injury severity and paw placement/sensorimotor issues.

In parallel, we characterized locomotor recovery in the three groups using the BBB locomotor scale and subscore. The moderate 150 kDyn animals showed improved locomotor recovery, both on the overall BBB and on the BBB subscore (Fig. 3). Together, these experiments provide important baselines for future locomotor and neuropathic pain testing using morphine/(+)-naltrexone.

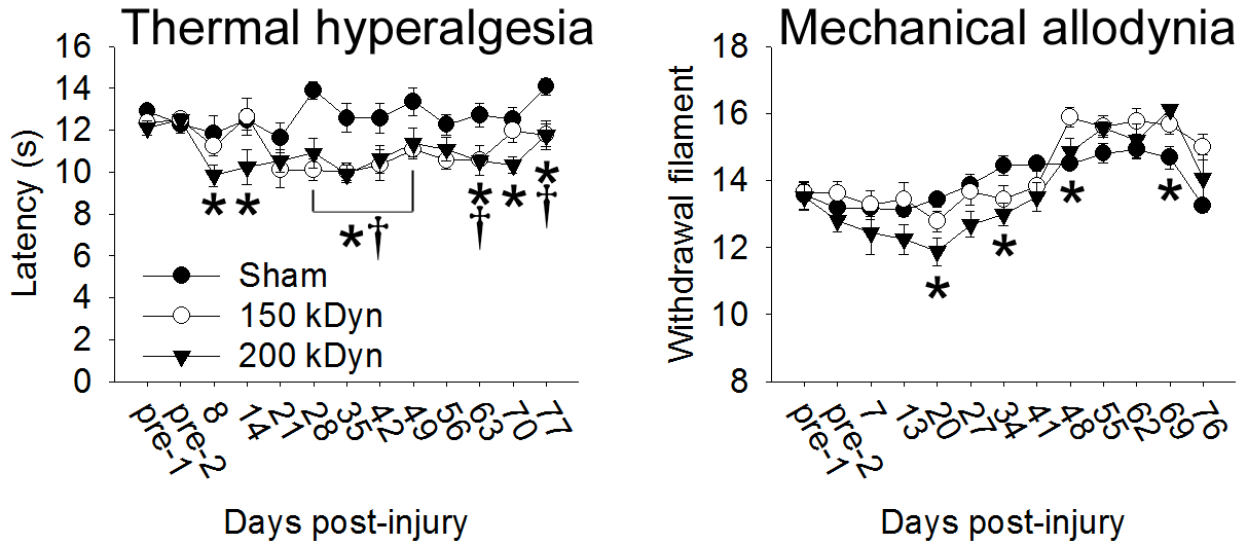

Fig. 2. SCI rats develop modest neuropathic pain symptoms. Rats were baseline tested for mechanical and thermal thresholds, then subjected to sham (laminectomy), moderate SCI, or severe SCI. Thermal and mechanical pain thresholds were assessed weekly after surgery. The withdrawal filaments reported here at 20 dpi correspond to ~5 g threshold for sham and to ~4.74 g for 200 kDyn (see Bonin et al., Mol. Pain 2014 PMID: 24739328). Both severe and moderate SCI animals displayed thermal hyperalgesia; only severe SCI animals had mechanical allodynia. Two-way repeated measures ANOVA with Holm-Sidak post-hoc. Asterisks indicate $p<0.05$ between sham and 200 kDyn; daggers indicate $p<0.05$ between sham and 150 kDyn.

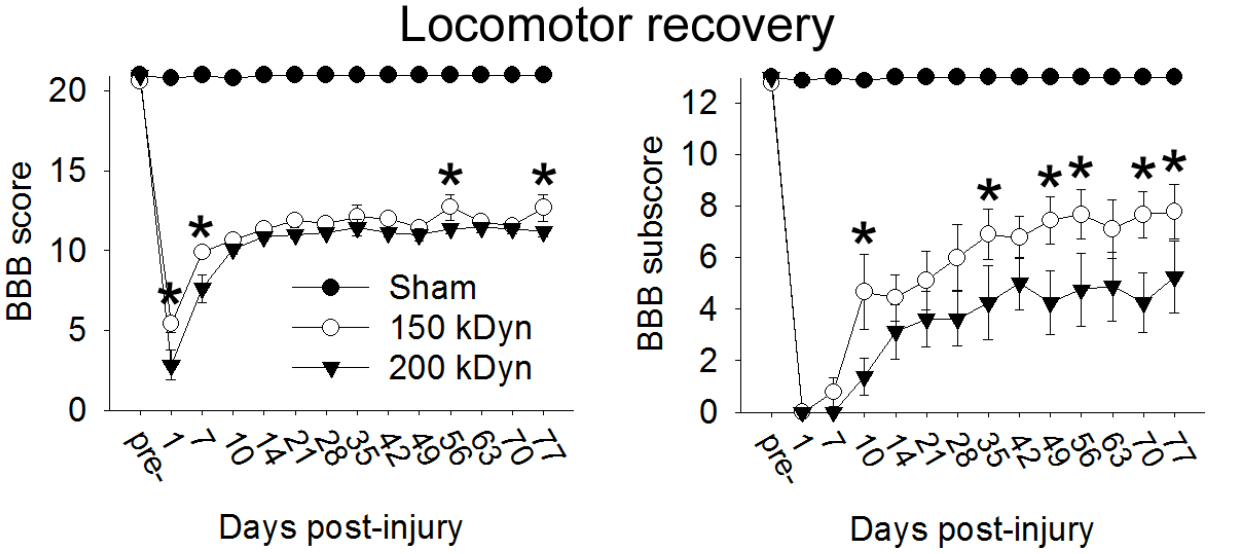

Fig. 3. Timecourse of locomotor recovery after sham surgery or moderate or severe SCI. On both overall BBB score and BBB subscore, 150 kDyn SCI rats showed significantly better locomotor scores overall (group difference) and at specific post-injury times compared to 200 kDyn rats. Two-way repeated measures ANOVA with Holm-Sidak post-hoc test. Asterisks indicate $p<0.05$ between 150 kDyn and 200 kDyn rats.

We found that these injuries caused neuropathic pain, although SCI-induced pain was not especially robust, and the 200-kDyn SCI animals had persistent sensorimotor deficits that complicated correctly identifying pain thresholds. Tissue from these animals has been collected/stored for histological and RNA expression analyses; further analyses will be pursued when interesting or necessary. These behavioral results built a strong foundation for starting another animal study to explore the effects of morphine in a clinically-relevant rat contusion SCI model.

Establishing effects of post-SCI morphine treatment in female rats

Ultimately, we will test whether (+)-naltrexone improves post-SCI morphine effects on constipation and neuropathic pain; however, first, we must establish a baseline model using our contusion device and morphine treatment. Our first contusion SCI experiments suggested that the severe SCI caused excess loss of sensation, which precluded measuring neuropathic pain thresholds. Therefore, we used a moderate contusion SCI model (150 kDyn force, 1 second dwell), which has been identified as a model for SCI-induced neuropathic pain[9]. We performed this preliminary study in female Sprague-Dawley rats, since male rats are more prone to bladder care issues (e.g., infection or bladder emptying issues).

Based on this previous research (citations above), we expected that moderate (150-kDyn force) or severe (200-kDyn force) midline contusion SCI could cause below-level neuropathic pain and bowel issues. Therefore, this experiment included four groups: sham/saline, sham/morphine, SCI/saline, and SCI/morphine (n=5-6 each). Female Sprague-Dawley rats were subjected to T9/T10 laminectomy, then were stabilized and raised slightly on the device's stereotaxic frame. SCI rats were leveled to ensure midline contusion, then placed under the Infinite Horizons impactor for contusion SCI (**Fig. 1**).

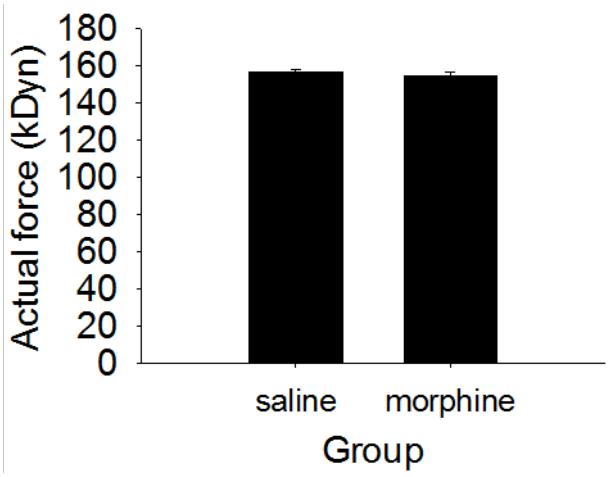

Fig. 1. Actual force of SCIs for rats in the saline and morphine groups. Saline = 157 ± 1 kDyn; morphine = 155 ± 2 kDyn.

For our initial post-SCI morphine treatment – which models clinical acute analgesic morphine treatment – saline or morphine (5 mg/kg) were injected 2x per day beginning 24 h post-SCI for 7 d. We hypothesized that SCI would cause neuropathic pain within several post-SCI weeks, and that morphine would exacerbate post-SCI pain. Further, we expected that morphine would cause constipation. Locomotor recovery with SCI/morphine was also examined.

Our neuropathic pain outcomes were mechanical allodynia (von Frey filament testing) and thermal hyperalgesia (Hargreaves plantar hindpaw heat) (**Fig. 2**). For behavioral tests, all experimenters are blinded to experimental group. Rats underwent pre-

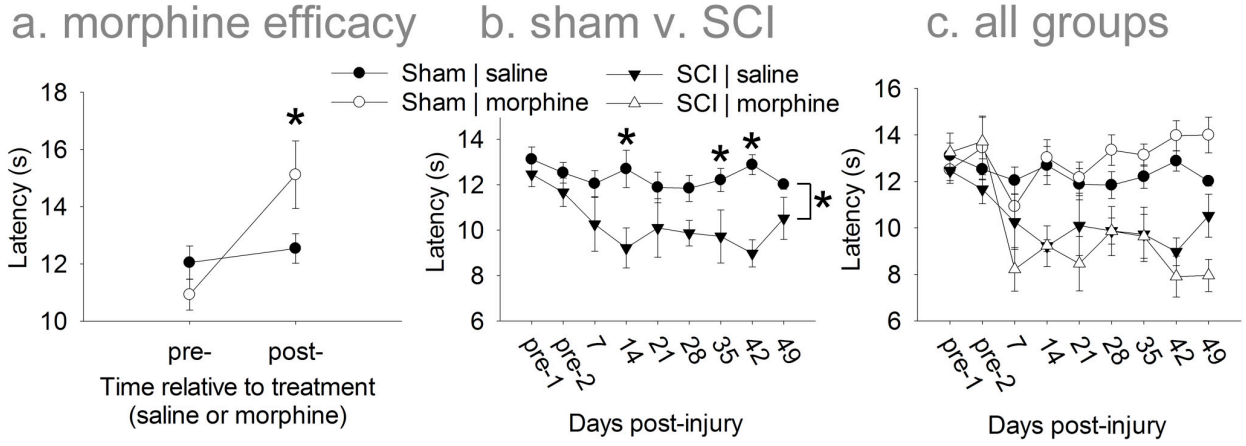

Fig. 2. SCI causes thermal hyperalgesia on the Hargreaves test; morphine relieves acute pain and does not exacerbate chronic pain in this SCI model. (a) Sham/morphine, but not sham/saline rats show analgesia 60 min after s.c. injection of saline or morphine (on 8 dpi; 7th day of saline/morphine treatment). Here, analgesia is represented by longer latency to response. (b) Sham versus SCI; saline groups only. SCI elicits thermal hyperalgesia. (c) We hypothesized that acute transient morphine treatment would exacerbate chronic SCI pain; however, morphine for 7 d post-SCI has little effect on chronic thermal nociceptive thresholds in this model.

surgical testing for nociceptive thresholds (2x for each von Frey and Hargreaves tests). Thresholds were tested weekly post-injury until the final timepoint (49 dpi). Acute morphine treatment effectively reduced thermal sensitivity (longer latency to response 60 min after morphine treatment on 7 d post-SCI; **Fig. 2a**).

As predicted, SCI-induced below-level thermal neuropathic pain was somewhat delayed and became apparent ~14-35 dpi. This was at least partly due to the lack of sensory and motor control at early times post-injury. Compared to sham/saline-treated animals, SCI/saline rats had significant thermal hyperalgesia (**Fig. 2b**; overall difference and differences at 14, 35, and 42 dpi). Acute morphine treatment for 7 d did not exacerbate long-term SCI-induced thermal hyperalgesia at the dosing paradigm tested in this initial study (**Fig. 2c**).

Mechanical allodynia was also assessed using von Frey filaments. Acute morphine treatment did not have as robust an effect on mechanical allodynia (**Fig. 3a**). This could be due to issues with the rats spending significant time on individual racks for fecal pellet collection. Having the same rats in hanging cages for fecal collection and for pain testing may also affect their pain thresholds – an unusual pattern of decreasing withdrawal filament thresholds was observed over the course

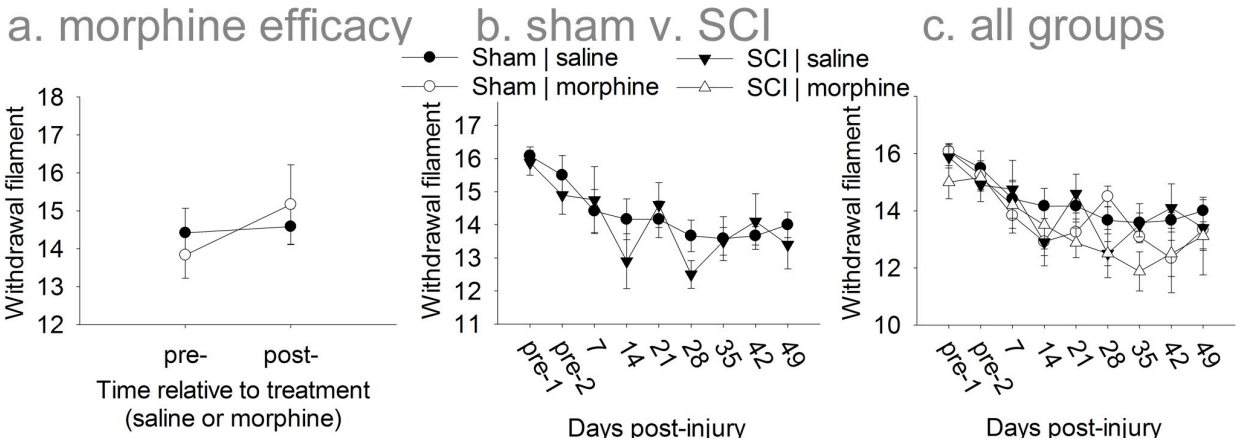

Fig. 3. Mechanical (von Frey test) thresholds were not significantly altered by SCI or morphine in this model. (a) Effects of acute morphine injection on mechanical nociceptive thresholds (pre- and 40 min post-morphine). (b) Sham versus SCI; saline groups only. SCI did not lower pain thresholds compared to sham animals. Note the decreasing thresholds even for control animals – sitting in the hanging wire cages for long periods (for pellet collection) likely sensitized rats' hindpaws to mechanical stimuli. (c) Morphine had no significant effect on sham or SCI thresholds.

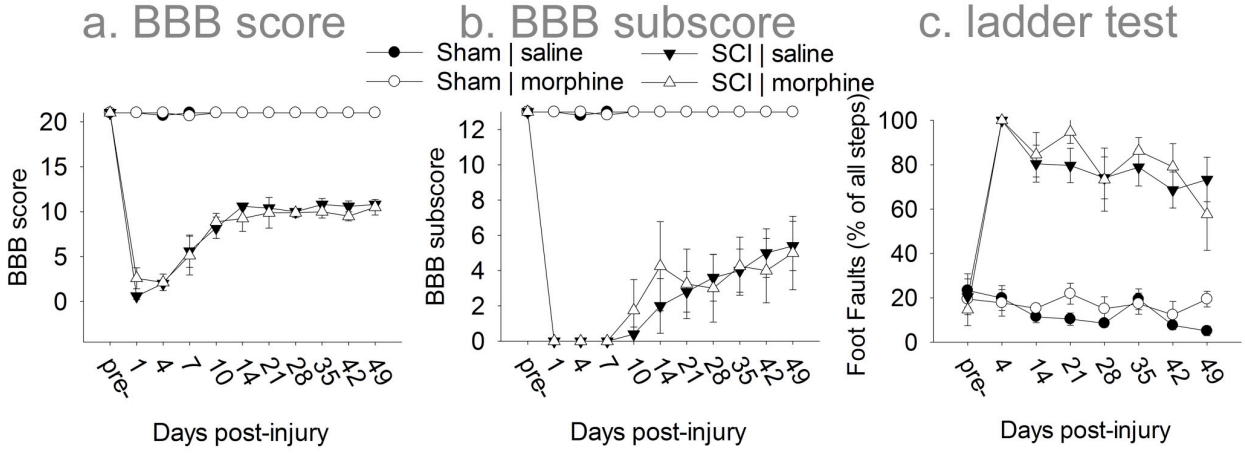

Fig. 4. Locomotor recovery after sham/SCI and treatment with saline or morphine. (a,b) SCI caused the expected locomotor deficits, as measured by BBB score (a) and subscore (b). (c) The horizontal ladder test was developed and optimized in our lab. Soon after injury, animals were unable to successfully step on the ladder; rats gradually recovered some stepping ability over time. Acute, 7d morphine treatment had no significant effect on locomotor recovery.

of the experiment. Future experiments could avoid this by having separate rats for pain testing and for fecal pellet collection. While noting this caveat, our data show that this moderate T9 contusion SCI did not induce robust mechanical allodynia (**Fig. 3b**) and morphine did not lower mechanical thresholds in chronically injured animals (**Fig. 3c**).

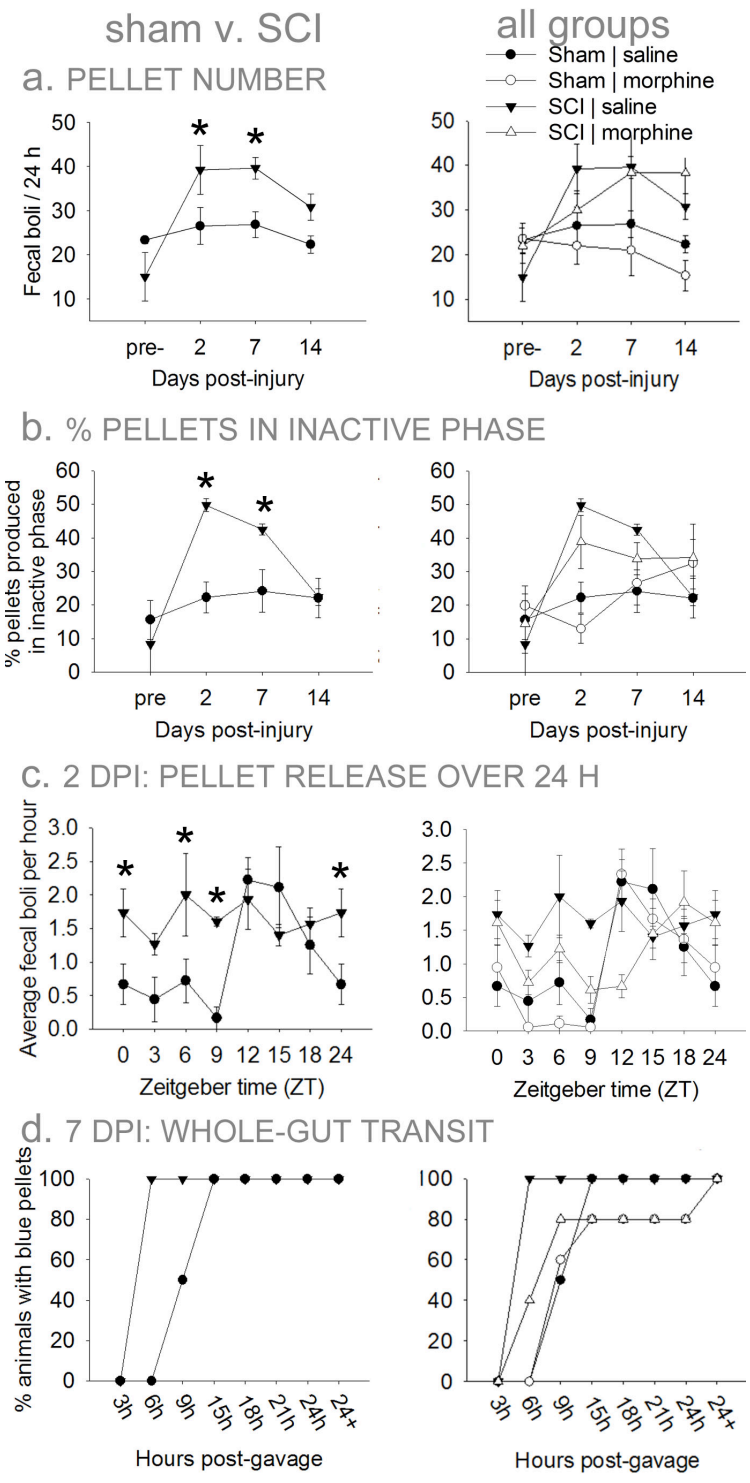

Fig. 5. SCI and morphine cause acute, transient bowel dysfunction. (a) SCI rats released more pellets than sham rats at 2 and 7 d post-SCI. Morphine had little effect. (b) SCI rats released more pellets at 2 and 7d post-SCI during the inactive phase. (c) At 2d post-surgery, SCI rats released more pellets at unusal times (inactive phase). (ZT0 = lights on; ZT12 = lights off.) (d) At 7d post-surgery, SCI rats had reduced whole gut transit time. Morphine caused some constipation in sham and SCI rats.

In parallel, we characterized locomotor recovery in the four groups using the BBB locomotor scale, BBB subscore, and newly-developed horizontal ladder test. Both saline- and morphine-treated SCI rats recovered similarly on the BBB and on the BBB subscore (**Fig. 4a,b**). In addition, the development and scoring of the horizontal ladder test in our lab has been achieved (**Fig. 4c**). Morphine-treated SCI rats performed and recovered similarly to control saline-treated SCI rats. (Percent foot faults are shown; another scoring system that accounts for foot fault severity [e.g., total miss; slip off rung, etc.] is also assessed.) Together, these experiments provide important baselines for future locomotor and neuropathic pain testing using morphine/(+)-naltrexone.

Finally, for the first time we studied bowel function after SCI in rats. As far as we can tell, no study has ever examined fecal production at various times after experimental rodent SCI. Developing a rodent model of SCI-induced bowel dysfunction could have important implications for identifying mechanisms and treatments for constipation and/or fecal incontinence. Here, several parameters were assessed: Whole gut transit time; fecal pellet number; fecal pellet weight; and fecal pellet production over the circadian cycle. These parameters were assessed over 24 h periods at 4 times: prior to surgery, and at 2, 7, and 14 d post-SCI. Rats were first gavaged (into the stomach with a feeding tube) with Evan's blue. Using this strategy, the time of first blue fecal pellet represents the "whole gut transit time" (time for the dye to get from stomach to pellet). 30 min after gavage, animals were placed in individual hanging cages

and fecal pellets were collected every 3 h for 24 h. Wet and dry pellet mass were assessed; also, total 24 h food and water consumption were recorded.

Interestingly, we found that SCI caused profound acute bowel dysfunction that largely resolved over two weeks. Fecal pellet production was increased in SCI-saline animals at 2 and 7 d post-SCI (Fig. 5a). Increased pellet production was mainly due to abnormal pellet release during the rats' inactive phase: Significantly more pellets were released by SCI rats during the inactive phase at 2 and 7 dpi. For instance, at 2 dpi, SCI-saline rats released ~50% of their total pellet number during the inactive phase (Fig. 5b). This is highlighted by the timecourse of pellet production from sham/SCI animals at 2 dpi (Lights on (inactive) from ZT0-12, off (active) from ZT12-24; Fig. 5c). Finally, whole-gut transit was studied by gavaging rats with Evan's blue dye and recording the time of first blue pellet appearance. Only 7 dpi is presented here (Fig. 5d). SCI rats had reduced whole-gut transit time soon after injury (leftward shift in graph), suggesting that they may have fecal incontinence. Although SCI rats released more fecal weight, they ate the same amount of food as sham controls (not shown). Together, these data suggest that SCI rats may have severe, transient circadian disruption and have decreased ability to absorb nutrients.

The effect of morphine on post-SCI fecal production was also examined. Morphine had no significant effect on overall post-SCI pellet production or timing, although the pattern of pellet release at 2 dpi was slightly more normal (Fig. 5c). As expected, morphine caused constipation in some animals (delayed production of blue pellets; Fig. 5d).

These results could have important implications for the study of post-SCI bowel dysfunction and circadian disruption. In follow-up experiments, we will study whether this occurs in male rats (see below) and how this relates to circadian disruption and nutrient deficiency.

Effects of SCI and morphine on pain and bowel dysfunction in male rats

The studies reported here were performed on female rats. It is possible that morphine-elicited chronic pain was not observed due to the use of females; also, it is increasingly apparent that sex differences have important implications for pain and pathology. In our ongoing study, male rats are being used to determine whether they experience SCI-induced neuropathic pain and constipation. The design of the study is based on the female study, but is improved to have better resolution for blue pellet identification. This study will establish whether SCI-induced incontinence also occurs in males, as well as whether circadian disruption of fecal output occurs. Results from this ongoing study will be presented in our next Progress Report.

KEY RESEARCH ACCOMPLISHMENTS

- Hired Dr. Gaudet, an experienced SCI researcher.
- Assembled contusion SCI device and began SCI experiments.
- Completed SCI experiments to develop SCI models of pain and neurogenic bowel disorder.

CONCLUSIONS

We have progressed in developing the clinically relevant contusion SCI model in our lab. Contusions at moderate and severe injury severities have been successfully completed, with associated locomotor and neuropathic pain assessments. Further, we have designed a new useful strategy for studying neurogenic bowel dysfunction after SCI: Rats are placed in hanging cages for 24 consecutive hours, and fecal pellets are collected over time. Whole gut transit was studied by gavaging the rats with Evan's blue dye, then recording the first appearance of blue pellets.

For neuropathic pain outcomes, injury models that were previously shown to cause below-level neuropathic pain (150 kDyn force bilateral contusion SCI at T9, 1 s dwell) [9] are not eliciting robust pain in our lab. Having a pain-eliciting injury model is critical for studying the chronic

effects of acute transient morphine treatment. Therefore, our next experiment will examine another reported model of rat neuropathic pain: unilateral 200 kDyn contusion at C5. This injury causes pain symptoms in the ipsilateral fore paw; pain thresholds in the hind paw will also be examined. In parallel, the same injury will be performed at T12/L1 to establish whether this causes neuropathic pain in the hind paw. Once an effective SCI-elicited pain model is established, experiments testing morphine and (+)-naltrexone will commence.

In the SCI-induced neurogenic bowel dysfunction study, acute post-SCI incontinence was observed for the first time. Rats showed increased pellet output (number and weight), circadian dysregulation of fecal output (abolished at 2 dpi; improved at 7 dpi; and typical at 14 dpi), and reduced whole gut transit time. Despite symptoms of neurogenic bowel, the rats ate the same amount of food as sham rats suggesting that they may have acute post-SCI nutrient imbalance. Similar patterns have been observed in females (described here) and males (analysis ongoing; presented next quarter).

As far as we can tell, early post-SCI bowel dynamics in the clinic or in a rat model have not been described, but in chronic SCI patients constipation and incontinence are both serious issues[5, 10]. There are also injury level-dependent effects on the dynamics of neurogenic bowel dysfunction. Injuries rostral to the lumbar spinal cord generally cause hyperreflexive colon, whereas injuries in the lumbosacral spinal cord can cause colonic paralysis[11]. In our next study, contusion SCIs will be performed at high thoracic (T3) or lumbar (L1) spinal cord and bowel function will be studied prior to injury and at 7 dpi and a chronic time (e.g., 42 dpi). This will allow us to establish whether constipation develops in chronically injured SCI rats, and whether injury level in this rat SCI model has an effect on bowel dynamics. Animal activity over the day at different times post-injury will also be studied to determine whether there is a more general SCI-induced circadian dysregulation or loss of homeostasis. If constipation is observed at chronic post-injury times, the effects of morphine and (+)-naltrexone can be tested. Thus, for the first time, we describe early post-injury bowel dysfunction in rats and have successfully developed a model for studying post-SCI neurogenic bowel disorder.

In summary, the past year has seen addition of a trained SCI researcher who has established the contusion SCI model in the lab. Future experiments will identify a functional SCI neuropathic pain model to test the effects of morphine and (+)-naltrexone. Further, we have created a system for studying SCI-induced neurogenic bowel disorder in rats – this may be the first time anyone has studied post-SCI bowel dynamics in rat. Future studies on neurogenic bowel in the lab could have broad application for the SCI field and for patients with SCI. In early 2016, we aim to wrap up studies to submit manuscripts to journals for publication.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a. Nothing to report
b. Nothing to report

INVENTIONS, PATENTS AND LICENSES

Nothing to report

REPORTABLE OUTCOMES

Nothing to report

OTHER ACHIEVEMENTS

The previous lead researcher on the project, Dr. Amanda Ellis, was hired for an industry position where she helps and advises physicians treating SCI patients with neuropathic pain.

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